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# **IL-12 and IL-4 activate a CD39-dependent intrinsic peripheral tolerance mechanism in CD8<sup>+</sup> T cells**

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**Abbreviations:** ATP, adenosine triphosphate; DC, dendritic cell; TIL, tumor-infiltrating lymphocytes; Treg, regulatory T cell.

## Abstract

Immune responses to protein antigens involve CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which follow distinct programs of differentiation. Naïve CD8 T cells rapidly develop cytotoxic T-cell (CTL) activity after T-cell receptor stimulation, and we have previously shown that this is accompanied by suppressive activity in the presence of specific cytokines, i.e. IL-12 and IL-4. Cytokine-induced CD8<sup>+</sup> regulatory T (Treg) cells are one of several Treg-cell phenotypes and are Foxp3<sup>+</sup> IL-10<sup>+</sup> with contact-dependent suppressive capacity. Here, we show they also express high level CD39, an ecto-nucleotidase that degrades extracellular ATP, and this contributes to their suppressive activity. CD39 expression was found to be upregulated on CD8<sup>+</sup> T cells during peripheral tolerance induction in vivo, accompanied by release of IL-12 and IL-10. CD39 was also upregulated during respiratory tolerance induction to inhaled allergen and on tumor-infiltrating CD8<sup>+</sup> T cells. Production of IL-10 and expression of CD39 by CD8<sup>+</sup> T cells was independently regulated, being respectively blocked by extracellular ATP and enhanced by an A2A adenosine receptor agonist. Our results suggest that any CTL can develop suppressive activity when exposed to specific cytokines in the absence of alarmins. Thus negative feedback controls CTL expansion under regulation from both nucleotide and cytokine environment within tissues.

## Introduction

CD8<sup>+</sup> T-cell responses are essential for controlling certain intracellular infections but can also mediate significant tissue damage and immunopathology. Large clonal expansions in antigen-specific CTL in vivo require multiple signals to be integrated via dendritic cell (DC):CD8<sup>+</sup> T-cell interaction and are usually rapidly downregulated [1, 2]. Although CD8 responses can be controlled by Foxp3-expressing CD4<sup>+</sup> regulatory T cells (Treg) [3], CD8<sup>+</sup> T cells themselves exhibit potent immunoregulatory activities in a variety of models and were originally described as “cytotoxic/suppressor T cells” [4]. In vitro differentiation of CD8<sup>+</sup> T cells can result in conventional “Tc1” cells in the presence of TCR signals alone, but Treg cell development in the presence of IL-4 and IL-12 [5]. These cytokine-induced CD8<sup>+</sup> Treg cells are Foxp3<sup>+</sup> and mediate contact-dependent suppressive activity in addition to IL-10 secretion [5]. However, a number of different models for CD8<sup>+</sup> Treg cell development have been proposed and their mechanisms of action remain unclear [6].

CD39 is constitutively expressed on CD4<sup>+</sup> Foxp3<sup>+</sup> Treg cells and represents an important pathway for negative regulation of inflammation [7]. It is a nucleotidase that breaks down extracellular ATP, which is released from necrotic cells or activated T cells and epithelium [8, 9]. Extracellular ATP is elevated in inflamed tissue and acts as an alarmin, activating dendritic cells and cellular immunity via P2 receptors [10, 11]. CD39 and CD73 degrade ATP to adenosine, which is tolerogenic and a ligand for the A2A receptor [12]. CD39 expression on CD4<sup>+</sup> Foxp3<sup>+</sup> Treg cells is known to contribute to their suppressive function [7, 13, 14] but how CD39 expression is regulated on Foxp3<sup>+</sup> T cells is not known.

In this study, we identify CD39 as a functionally important marker for CD8<sup>+</sup> Treg cells and demonstrate an alternative pathway for CD8<sup>+</sup> T-cell activation that upregulates

CD39 but not memory markers in response to cytokines during tolerance induction in vivo. Our data indicate that suppressive functions are intrinsic to conventional CD8<sup>+</sup> T-cell populations and their expression is regulated by purinergic signals including ATP and adenosine. This study establishes CD39 as a mediator of CD8<sup>+</sup> Treg cell function under dynamic control by the CTL environment and demonstrates a novel pathway of peripheral tolerance that is independent of Foxp3.

## Results

### *CD39 is induced on differentiating CTL by IL-12 and IL-4*

Stimulation of CD8<sup>+</sup> T cells in the presence of specific cytokines has previously been shown to induce a Treg cell phenotype associated with capacity for copious IL-10 production and Foxp3-independent suppressive capacity [5]. In Fig. 1A, we analyzed in vitro-induced CD8<sup>+</sup> Treg cells from OT-I mice for expression of CD39. The data showed that addition of either IL-12 or IL-4 to cultures increased the capacity for IL-10 production after restimulation of differentiated CD8<sup>+</sup> effector cells, and they were most effective when added together, as previously shown. We found that CD39 surface expression was also upregulated by these cytokines, and correlated with IL-10 intracellular staining. In conventional “Tc1-type” effectors differentiated in the absence of IL-4 and IL-12, IFN- $\gamma$  production was present but CD39 was not expressed. Addition of IL-12 and IL-4 both induced expression of T-bet, a CTL-associated transcription factor, and further upregulated levels of granzyme B within cells consistent with their cytotoxic function. A time course of CD39 upregulation with or without IL-12 + IL-4 was performed (Fig. 1B), which revealed that at day 2 of differentiation, low levels of CD39 were expressed in both Tc1 and Treg cell cultures. However, by day 4, Treg cells expressed maximal CD39 while Tc1 cells had lost CD39 expression altogether. In further experiments OT-I cells were cultured for 4 days as this achieved the most highly significant difference in CD39 expression between cell types. CD73 expression was also upregulated on differentiated CD8<sup>+</sup> T cells, compared to fresh cells which were CD73<sup>-</sup>, however there was no difference in CD73 expression between Tc1 and Treg cells (Fig. 1C). The phenotype of Tc1 and Treg cells was stable after a secondary restimulation with anti-CD3/28 beads and culture in the same or opposing cytokine conditions (Fig. 4D).

### *IL-10 and CD39 are differentially regulated by ERK signaling and extracellular ATP*

Since extracellular ATP is a substrate for CD39 enzymatic activity and is present in inflamed tissue, we examined the effect of ATP on Treg cell development in vitro using a stabilized derivative – ATP- $\gamma$ -S, an agonist for the P2X7 receptor. Addition of ATP- $\gamma$ -S to Tc1 cultures reduced cell viability considerably, but in Treg cell cultures viability was maintained while capacity for IL-10 production was dramatically curtailed (Fig. 2A&B). By contrast, IFN- $\gamma$  synthesis was maintained in the presence of ATP- $\gamma$ -S and staining intensity in positive cells was increased, suggesting enhanced IFN- $\gamma$  synthesis. CD39 expression in developing Treg cells was unaffected by ATP- $\gamma$ -S, suggesting differential regulation of CD39 and IL-10. To confirm this possibility we added U0126, an ERK MAP kinase inhibitor to the cultures, since ERK intracellular signaling is known to induce IL-10 production in CD4 T cells [15]. We found that ERK inhibition also blocked IL-10 production capacity in CD8<sup>+</sup> Treg cell cultures, but had no effect on IFN- $\gamma$  and significantly enhanced CD39 expression (Fig. 2C). This confirmed that distinct signaling pathways must control expression of CD39 and IL-10 in Treg cells.

### *Suppression by IL-12+IL-4-induced CD8 Treg cells is CD39-dependent and correlates with ATP breakdown*

To test whether CD39 was functionally important in CD8<sup>+</sup> induced Treg cells we performed suppression assays in the presence or absence of the CD39 inhibitor ARL67156, which blocks breakdown of ATP by CD39 [16]. OT-I CD8<sup>+</sup> Treg cells generated as above for 4 days were restimulated with freshly isolated OT-I lymph node/spleen cells labeled with CFSE to assess target cell proliferation. % suppression was calculated relative to proliferation in the absence of added cells. Titration of Tc1 and Treg cells (Fig. 3A) confirmed the potent suppressive function of

Treg cells but not Tc1, with suppression seen at effector:target ratios of 1:8 and above. Addition of ARL67156 largely abrogated suppression mediated by Treg cells. Pooled data expressed as % cell division (Fig. 3B) revealed that the effect of ARL67156 was not due to an ability to enhance T-cell proliferation, since there was a trend for less proliferation of isolated target cells in the presence of the inhibitor. However the reduced target cell division in the presence of Treg cells was significantly abrogated by ARL67156, indicating that CD39 activity on Treg cells is largely responsible for their suppressive activity.

We then assessed the ability of Tc1 and Treg cells to degrade extracellular ATP, by pulsing differentiated and washed Tc1 or Treg cells with 100 $\mu$ M ATP, and removing supernatants after 0-60 minutes of culture. Supernatants were analyzed for breakdown products by HPLC (Fig. 3C). The data showed that Treg cells break down ATP and ADP significantly more rapidly than Tc1 cells, as expected from their CD39 expression levels, and convert it to AMP, which would be further converted to adenosine by CD73 [17]. Adenosine itself could not be detected by HPLC. Since adenosine can be rapidly converted to inosine, a pro-inflammatory molecule [18], we also pulsed cells with adenosine and analyzed supernatants for inosine, however no difference in inosine release was detected between the two subsets. Together our data suggest CD8<sup>+</sup> Treg cells act through conversion of ATP to adenosine via the activity of CD39 alongside CD73.

#### *Systemic CD8<sup>+</sup> T-cell tolerance in vivo involves an alternative CD39 activation pathway*

We then determined whether CD39 was a marker of Treg cell induction in vivo, in a model of systemic tolerization in OT-I mice. Exposure of TCR transgenic animals to low levels of cognate peptide has been shown to result in non-deletional T-cell



tolerance [19]. We used a similar protocol to tolerize OT-I mice by repeated dosing with 2 $\mu$ g OVA<sup>257</sup> peptide. Injection of higher doses of peptide, or low doses combined with an adjuvant, causes rapid death of mice by a toxic shock-like syndrome (not shown). With 2 $\mu$ g dosing however, no ill-effects were seen at any time point. Moreover, circulating CD8<sup>+</sup> T cells in these animals expressed high levels of CD39 by day 7 of tolerization, and maximal levels by day 10 (Fig. 4A&B). This did not reflect a conventional pathway of T-cell activation since CD39 expression was not accompanied by increased CD44 and decreased CD45RB expression. This was not due to insufficient antigen dose since a substantial proportion of blood CD8<sup>+</sup> cells lost CD62L, the lymph node homing receptor. These data are consistent with previous findings in vitro, showing retention of naïve markers but loss of CD62L in developing Treg cells [5].

To confirm that systemic T-cell tolerization had occurred in animals with high T-cell CD39 expression, splenocytes were prepared from tolerized animals (day 10) and naïve control OT-I mice. These were CFSE-labeled and stimulated with 0.2  $\mu$ g/ml OVA<sup>257</sup> peptide in vitro (Fig. 4C). A proliferative response was seen in control splenocytes but those from tolerized animals failed to divide, indicating profound unresponsiveness or anergy in T cells. This effect was evident over a wide range of peptide concentrations (not shown).

#### *IL-12 and IL-10 are involved in systemic T-cell tolerance induction*

We tested whether production of IL-12 could be involved in peripheral tolerance induction in vivo. OT-I mice were tolerized as in previous experiments (Fig. 4) but were given 0.5mg anti-IL-12 neutralizing antibody or IgG control on days 0, 2 and 4. CD39 expression on circulating CD8 T cells was monitored (Fig. 5A), and this revealed delayed CD39 upregulation in mice treated with anti-IL-12, since CD39

levels were significantly lower than controls on day 4. CD39 was not significantly repressed at later time points. This might not have been due to insufficient neutralization of IL-12 since increased T-bet expression was apparent in tolerized mice but this was largely abrogated at day 10 in anti-IL-12-treated animals (Fig. 5B). Foxp3 was not expressed in tolerized CD8<sup>+</sup> T cells (Fig. 5B). To confirm a role for IL-12 in this model we measured serum IL-12 p70 in the same animals (Fig. 5C). IL-12 was only detectable at day 7. By contrast IL-10 was detected in the serum at all time points post-tolerization and peaked at day 10. These distinct kinetics are consistent with the hypothesis that IL-12 produced by dendritic cells during cognate interaction with T cells induces differentiation of IL-10-producing CD39<sup>+</sup> CD8 cells.

#### *Adenosine receptor signaling enhances CD39 levels during peripheral tolerance induction*

Since CD39 activity was implicated in the OT-I tolerance model, we asked whether adenosine, the breakdown product of ATP, would also contribute to this pathway. We used a stable agonist of the A2A adenosine receptor, expressed on T cells [7]. CGS21680 A2A agonist [20] was administered to OT-I mice alongside OVA<sup>257</sup> peptide and CD39 levels on circulating CD8<sup>+</sup> T cells were monitored (Fig. 6). The results showed enhancement of CD39 expression in CGS21680-treated mice compared to animals given diluent alone; this was significant at days 7 and 10. Neither the A2A agonist or adenosine showed a similar effect in in vitro differentiation cultures (not shown). P2X7 agonists could not be used in the in vivo model due to their adjuvanticity.

#### *Respiratory and tumor-associated tolerance to ovalbumin involves upregulated CD39 on CD8<sup>+</sup> T cells*

Since tolerization of OT-I mice with peptide restricts clonal expansion of responding T cells, we examined CD39 expression in non-transgenic mouse models of tolerance to ovalbumin. C57BL/6 mice can be tolerized by intranasal administration of whole OVA protein in the absence of adjuvant [21]. To test whether CD8<sup>+</sup> T cells were involved, we administered OVA to naïve mice intranasally and phenotyped airway-infiltrating T cells after 6 days (Fig. 7A). As expected, OVA challenge recruited CD4<sup>+</sup> T cells into the airway, however we found that equal numbers of CD8<sup>+</sup> T cells were recruited. Furthermore, expression of CD39 was highly upregulated on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells after OVA challenge, suggesting involvement of both CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells. We also extracted T cells from lung tissue and draining lymph node of tolerized C57BL/6 animals, and found that CD39 was higher on tissue-resident CD8<sup>+</sup> cells compared to lymph nodes and was greatly induced by tolerization (Fig. 7B). These data suggest that CD39 is a useful marker for Treg cell induction ex vivo and implicate CD8<sup>+</sup> Treg cells in peripheral tolerance to inhaled protein antigens.

Heterologous antigens expressed in tumor cells are also known to induce T-cell tolerance due the suppressive environment propagated by the tumor. We therefore grew B16 melanoma cells, engineered to express OVA, in C57BL/6 animals and extracted tumor-infiltrating lymphocytes (TIL) and unaffected lymph node cells when tumors had developed subcutaneously (before metastasis). Tumor growth rates were similar to untransfected B16 cells (not shown). Compared to lymph node T cells, both CD4<sup>+</sup> and CD8<sup>+</sup> TIL from B16.OVA tumors expressed extremely high CD39 levels, consistent with a Treg cell phenotype.

## Discussion

Whilst the important role of thymically-committed, natural CD4<sup>+</sup> Treg cells in peripheral immune tolerance has been well established, certain immunoregulatory molecules can also be induced in developing effector T cells, including Foxp3 and IL-10 [15, 22, 23], thus allowing negative feedback inhibition of T-cell responses. This study demonstrates that CD39, a B-cell-associated molecule previously shown to be critical in natural Foxp3<sup>+</sup> Treg cell function [7, 13, 14], is readily induced on CD8<sup>+</sup> T cells by pro-inflammatory cytokines and mediates potent suppressive activity. The mechanisms used by CD8<sup>+</sup> Treg cells have remained controversial since the 1980s. Although CD8 populations do contain a natural Treg cell subset expressing CD122 [24], various subsets of induced CD8<sup>+</sup> Treg cells have also been described, while the original characterization of CD8<sup>+</sup> T cells ascribed them a “suppressor/cytotoxic” hybrid phenotype. The latter nomenclature fits the data we describe here, since we show that large proportions of a monoclonal CD8 population can switch to a regulatory phenotype when exposed to certain cytokines. Furthermore, two key suppressive molecules, IL-10 and CD39, are under distinct regulation and could therefore be expressed on different populations of Treg cells in different environments. CD73 by contrast, was highly expressed on both induced Treg cells and conventional Tc1 effectors. In addition to showing that a CD39 inhibitor reverses in vitro suppressive activity by cytokine-induced CD8 Treg cells, we examined purine catabolism in the extracellular milieu of Tc1 and Treg cells, which confirmed their distinct functions. Treg cells degraded ATP and its immediate breakdown product ADP more rapidly than control Tc1 effectors with low CD39 expression. Furthermore, Treg cells produced AMP more rapidly than Tc1 cells suggesting they could release adenosine. However further breakdown products of AMP could not be detected. We also pulsed cells with adenosine and detected resulting inosine, a pro-inflammatory product of adenosine deaminase [18]. Inosine was equally produced by both Tc1 and

Treg cells but at very low levels. Together the data suggest that modulation of the balance between ATP and adenosine is likely to play a key role in the regulatory function of these Treg cells. CD39-knockout studies, as performed in Foxp3<sup>+</sup> Treg cells [7], would be required to confirm the functional role of CD39.

The Treg cell phenotype described here is unusual in that it lacks memory and effector markers such as high CD44 expression and loss of CD45RB. We observed this in our in vivo tolerance model, but in our previous study we also showed this “naïve effector” phenotype is a feature of in vitro-induced CD8<sup>+</sup> Treg cells [5]. IL-4 and IL-12 treatment does not induce a similar phenotype in CD4<sup>+</sup> cells, but results in conventional memory effectors with a Th0 cytokine profile [5]. The inability of CD8<sup>+</sup> T cells to switch to a memory phenotype was not due to incomplete TCR ligation in our TCR transgenic model, since both CD39 and a loss of CD62L, the lymph node homing marker, were observed on substantial proportions of circulating T cells. This suggests that CD8 cells were migrating from lymphoid tissues into peripheral tissues in order to maintain tolerance by exerting suppressive function. This is supported by our observation that T cells from tolerized mice were profoundly unresponsive to TCR-mediated stimulation ex vivo, consistent with our previous in vitro observations of an anergic/suppressive phenotype in IL-12/IL-4-induced CTL [5]. CD25 expression was not observed in the in vivo model (data not shown), despite in vitro-derived CD8 Treg cells expressing CD25.

The pathway of peripheral tolerance described here is to our knowledge novel, since it involves the production of IL-12, a potent CTL expansion and differentiation factor. Peak IL-12 release in tolerizing mice was seen at day 7, while serum IL-10 levels peaked at day 10. This is consistent with our hypothesis that IL-12, which can be elicited from dendritic cells by CD8<sup>+</sup> T cells during cognate interaction [26], was responsible for induction of CD39<sup>+</sup>, IL-10-producing CD8s in vivo. In vivo IL-12 neutralization experiments confirmed this, although CD39 expression was only

delayed, not prevented, by anti-IL-12 therapy. By contrast, T-bet upregulation was completely abrogated by anti-IL-12, suggesting neutralization was effective. Previous work on CD4<sup>+</sup> Treg cells has shown high expression of T-bet in Foxp3<sup>+</sup> cells in Th1 environments [27], perhaps reflecting exposure to IL-12, and it has long been known that IL-12 induces IL-10 production in CD4<sup>+</sup> T cells [28]. Since anti-IL-12 had a limited effect in our in vivo model it thus seems likely that other cytokines such as IL-4 were also involved in Treg cell induction, despite the fact that CD8<sup>+</sup> cells rarely produce IL-4. Preliminary data suggest that IL-27 may also be able to enhance CD8<sup>+</sup> Treg cell development (our unpublished observations). Both IL-27 and IL-4 can be produced by dendritic cells [29, 30] and may have contributed to the alternative activation pathway we describe in OT-I mice.

Although the OT-I tolerance model we employed did not reflect normal T-cell clonal expansion due to the monoclonal nature of the peripheral T cells, our data also confirm that CD39 is greatly upregulated during respiratory and tumor-associated immune tolerance to OVA in wild-type animals. Indeed, despite CD4<sup>+</sup> T cells generally dominating the lung T-cell response to inhaled allergens, we found equal numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrated the airways after intranasal OVA treatment. Since Foxp3<sup>+</sup> CD4<sup>+</sup> Treg cells express CD39 and are known to mediate respiratory tolerance, it was not surprising to observe upregulated CD39 on airway-infiltrating CD4<sup>+</sup> cells. However the equally high levels seen on CD8<sup>+</sup> T cells strongly suggests CD8<sup>+</sup> Treg cells contribute to respiratory tolerance, presumably after allergen cross-presentation to CD8<sup>+</sup> cells via MHC class I [31]. Similarly, tolerance to tumor-associated antigens has been thought to be mediated by Foxp3<sup>+</sup> CD4<sup>+</sup> Treg cells, which have a dominant presence in TIL and allow tumors to escape rejection [32]. Our model utilized B16 melanoma cells transfected with OVA, an immunogenic heterologous antigen. Despite this, B16-OVA cells grow aggressively in C57BL/6 mice, like untransfected cells. We found equally high CD39 expression on CD4<sup>+</sup> and

CD8<sup>+</sup> TIL from B16-OVA tumors, suggesting collaboration between CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells in tolerance to tumor-expressed OVA. The results agree with data from a human study which demonstrated CD8<sup>+</sup> Treg cells expressing CD39 in TIL [33]. These observations are important since the pathway of CD8<sup>+</sup> Treg cell induction we describe differs markedly from that ascribed to Foxp3<sup>+</sup> Treg cell induction, and thus needs to be considered in strategies to manipulate immune tolerance in inflammatory or malignant disease.

The data generated in our in vivo tolerance model suggest that TCR ligation and cytokines alone are insufficient to elicit full differentiation of CD8<sup>+</sup> T cells into pro-inflammatory, effector T cells, despite previous work indicating a key role for IL-12 as a “third signal”, after TCR ligation and costimulation, for CTL expansion and differentiation [34]. The additional factor(s) required for effector differentiation can be provided by adjuvants, since active immunization of TCR-transgenic mice causes lethal immune activation. We show here that alarmins, produced by endogenous tissue under conditions of activation or stress, are potential factors required for CD8<sup>+</sup> effector development in addition to cytokines. ATP- $\gamma$ -S, a stabilized ATP that is recognized by P2 receptors including the P2X7 expressed by T cells and dendritic cells, blocked development of IL-10-producing CD8<sup>+</sup> cells in vitro without affecting CD39 expression. IL-10 production in CD4<sup>+</sup> T cells is dependent on ERK signalling [15], and we found this to be also the case in CD8<sup>+</sup> T cells, again without affecting CD39 expression. By contrast, an A2A receptor agonist, which mimics the effect of adenosine (the tolerogenic breakdown product of ATP) enhanced CD39 upregulation on CD8<sup>+</sup> cells in vivo during tolerization whilst not affecting IL-10 secretion. This was not observed in in vitro cultures, perhaps due to accumulation of endogenous adenosine in static cultures masking effects of the A2A agonist. These data suggest that CD39 and IL-10 are subject to differential regulation despite both mediating immunoregulatory functions. CD39 expression was relatively stable on in vitro

derived Treg cells when IL-4 and IL-12 were removed from the culture, whereas IL-10 expression was unstable[5]. This would allow dynamic control of CTL expansion and contraction in response to distinct environmental cues. Overall our data show that an intrinsic, Foxp3-independent program of differentiation mediates suppressive activity in developing CTL, in response to cytokine presence but absence of alarmins. This would assist development of peripheral tolerance in the presence of immunogenic antigens that do not cause tissue damage.



## Materials & Methods

**Mice:** OT-I TCR-transgenic mice [35], expressing TCR specific for OVA<sup>257-264</sup> (SIINFEKL, OVA<sup>257</sup>) and H-2K<sup>b</sup>, on a C57BL/6 genetic background were bred in our facility and used at 4-12 weeks. Male and female animals were used and all experimental groups were matched for age and sex. Wild-type C57BL/6 female mice were purchased from Harlan UK (Bicester, UK) and used at 4-12 weeks. Experimental procedures were performed under UK Home Office authorization with approval from our institutional animal welfare committee.

**Tolerization protocols:** OT-I mice were systemically tolerized by repeated i.p. injection of 2µg OVA<sup>257</sup> peptide in 100µl PBS, on days 0,1,2,3,4 and 7. For A2A receptor agonist treatment, CGS21680 (Tocris Bioscience, 100mM in DMSO) was diluted in PBS and injected separately i.p. at 25µg/mouse. Control animals were given diluent (DMSO in PBS) alone. In IL-12 neutralization experiments 0.5mg anti-IL-12 (clone C17.8, BioXCell, West Lebanon, NH) or rat IgG control (Sigma) was injected i.p. in PBS on days 0, 2 and 4. For respiratory tolerance in C57BL/6 mice, animals were given 50µg OVA (Grade V, Sigma) in PBS i.n. on days 0,1,2 and 3. Mice were killed on day 6 and bronchoalveolar lavage performed with 1ml PBS. Lungs were excised, cut into pieces and digested with collagenase (type IV, Sigma, 0.5 mg/ml in DMEM + 10% FCS) for 45 minutes before release of lung cells by pipetting.

**Tumor growth:** OVA-transfected B16 melanoma cells (gift of Farzin Farzaneh) were grown to exponential phase in DMEM + 10% FBS + 2.5 mg/ml G418 (Sigma). Cells were harvested from flasks with a cell scraper, washed in PBS and  $2 \times 10^5$  cells injected s.c. in 100µl PBS into the dorsa of C57BL/6 recipients. Animals were killed approximately 1 month later when tumors were approximately 1cm diameter. Excised

tumors were collagenase digested as above, filtered through cotton wool and stained for analysis of tumor-infiltrating lymphocytes (TIL) by flow cytometry.

**T-cell culture:** OT-I lymph node and spleen single cell suspensions were prepared and cultured at  $1 \times 10^6$ /ml in XVIVO-15 serum-free medium (Lonza, Blackley, UK), 200 $\mu$ l volumes in 96-well plates, 37°C 5% CO<sub>2</sub>. OVA<sup>257</sup> peptide (Proimmune, Oxford, UK), ATP- $\gamma$ -S (125 $\mu$ M, Sigma) and U0126 (2.5 $\mu$ M, Calbiochem, Nottingham, UK) were added alongside diluent controls. After 4 days cells were washed in PBS + 1% FBS, restimulated with 20ng/ml PMA + 400ng/ml ionomycin + 3 $\mu$ M monensin (all Sigma) for 5 hours, then intracellular cytokine staining performed as previously described [36]. For crossover experiments, cells cultured with or without IL-4 and IL-12 for 5 days were Ficoll to remove dead cells and restimulated with 10 $\mu$ l anti-CD3/28 Dynabeads in the same or opposing cytokine conditions for a further 5 days. Suppression assays were performed as previously described [5] using OT-I CD8 T cells differentiated into Tc1 (OVA<sup>257</sup> only) and Treg cells (OVA<sup>257</sup> + IL-12 10ng/ml + IL-4 10ng/ml) for 4 days in 3ml cultures. These effector cells were washed and added to fresh CFSE-labeled (Life Technologies, Paisley, UK) OT-I target cells stimulated for 3 days with OVA<sup>257</sup> (2 $\mu$ g/ml), with or without ARL67156 (250 $\mu$ M, Sigma), before flow cytometry.

**ATP catabolism assays:** Tc1 and Treg cells generated as above were centrifuged over Ficoll to remove dead cells, washed in PBS, counted and re-cultured in serum-free RPMI medium,  $1 \times 10^6$ /ml. Either ATP or adenosine (Sigma) was added at 100 $\mu$ M to pre-warmed cultures. Samples were then removed after 0, 15, 30 and 60 minutes of culture. Samples were treated with an equal volume of 10% trichloroacetic acid to remove protein/cells, centrifuged at 12000g for 5 minutes and supernatants stored at -80°C. Nucleotide analyses were performed using a Waters trimodular HPLC system with photodiode array. The nucleotides were separated by anion-exchange on a Thermo Hypersil APS-2(250x3mm) 5u column, running a linear gradient from 100%

Buffer A (5mm KH<sub>2</sub>PO<sub>4</sub> pH 3.2) to 70% Buffer B (0.5M KH<sub>2</sub>PO<sub>4</sub> pH 3.5) over 25mins. Peaks were identified by retention time and spectrum. 10ul of sample was injected. Inosine analysis was performed on a Water 2690 HPLC system with photodiode array. 10ul samples were injected onto a Phenomenex Hyperclone ODS (C18) (150x4mm) 5u column, running an isocratic method with a 40mm ammonium acetate with 5mm tetrabutylammonium acetate buffer pH 2.75.

**Flow cytometry:** Cell surface staining was performed with approximately 1x10<sup>6</sup> cells and 0.1µg antibodies in 100µl PBS+1% FBS. Antibodies used were: anti-IFN-γ-PE/Cy7 (XMG1.2), anti-IL-10-APC (JES5-16E3), anti-CD39-PE (24DMS1), anti-T-bet-PE (eBio4B10), anti-granzyme B-eFluor660 (NGZB), anti-CD44-PE (IM7), anti-CD45RB-FITC (C363.16A), anti-CD62L-PE/Cy7 (MEL-14), anti-Foxp3-eFluor660 (FJK-16s), anti-CD4-FITC (RM4-5), all from eBioscience (Hatfield, UK) and anti-CD8β-APC (53-5.8, BioLegend, London, UK). Blood was collected from the tail into sodium citrate anticoagulant (Sigma) and 50µl stained directly with fluorochrome-labeled antibodies for 15 minutes prior to erythrocyte lysis with 0.5ml lysis buffer at room temperature (Sigma, 10 minutes). Intranuclear staining for T-bet and Foxp3, and granule staining for granzyme B, were performed as described [37]. For CFSE dilution assays lymph node and spleen cells were washed twice in PBS, labeled with 2.5µM CFSE at 37°C for 10 minutes and washed in PBS + 1% FBS before culture. Analysis was performed using a FACScalibur™ flow cytometer and CellQuest™ software (BD Biosciences, Oxford, UK); gating strategies shown in supplementary Figures 1&2.

**Cytokine ELISA:** IL-10 and IL-12 in mouse serum samples were measured using eBioscience Ready-Set-Go™ kits according to manufacturer's instructions.

**Statistical analysis:** GraphPad Prism 5 software (GraphPad, San Diego, CA) was used to plot and analyze the data. For in vitro experiments, data were analyzed using

paired t tests. For in vivo experiments with groups of animals, unpaired t tests were used except where significant differences between variances were observed, in which case Mann-Whitney tests were employed.

## **Acknowledgements**

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## **Conflict of interest**

The authors declare no commercial or financial conflict of interest.

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## Figure Legends

**Figure 1.** IL-4 and IL-12 induce differentiation of CD8 T cells that produce high levels of IFN- $\gamma$  and IL-10 and express CD39, granzyme B and T-bet. (A-D) Lymph node/spleen cells from OT-I TCR transgenic mice were stimulated with peptide  $\pm$  IL-4, IL-12 or both. (A) Cells were then restimulated with PMA/ionomycin for intracellular cytokine staining or stained directly for granzyme B and T-bet in cytoplasmic granules and nucleus respectively. Cells were analyzed by flow cytometry and CD8<sup>+</sup>-gated events are shown. Representative staining from one experiment of 4, analyzed at day 4. (B) Time-course of CD39 expression on developing CTL without ("Tc1"), or with IL-4 + IL-12 ("Treg") added to culture. Data are shown as mean $\pm$ SEM and are pooled from 4 independent experiments. Statistical comparisons between Tc1 and Treg cell samples are indicated. (C) CD73 staining of Tc1 (grey line), Treg cells (filled histogram) and freshly isolated CD8 cells from OT-I mice (dotted line); representative staining from 4 independent experiments. (D) Crossover experiment to determine stability of CD39 expression. Tc1 and Treg cells (day 5) were restimulated in secondary cultures with (Treg conditions) or without (Tc1 conditions) IL-4 and IL-12 as indicated. CD39 expression was analyzed after a further 5 days culture. Data are shown as mean $\pm$ SEM and are pooled from 4 independent experiments. \* $p < 0.05$ , \*\*  $p < 0.005$ ; paired t test; NS, not significant.

**Figure 2.** Differential regulation of surface CD39 expression and capacity for IL-10 production in CD8<sup>+</sup> T cells. (A) OT-I spleen cells were cultured in Tc1 (upper panel, peptide alone) or Treg-inducing (IL-4 + IL-12, lower panel) conditions, with or without addition of stabilized ATP- $\gamma$ -S. After 4 days cells were restimulated for intracellular cytokine staining. CD8<sup>+</sup>-gated events are shown. (B) IL-10, IFN- $\gamma$  and CD39 expression was measured in OT-I cells treated as described in panel A. Data are

shown as percentages and are shown as mean+SEM (n=4) pooled from 4 independent experiments. (C) OT-I spleen cells were cultured as described in panel B  $\pm$  UO126 ERK inhibitor. IL-10, IFN- $\gamma$  and CD39 expression was measured by flow cytometry. Data are shown as percentages and are shown as mean+SEM (n=4), pooled from 4 independent experiments. \*: p<0.05, \*\*:p<0.005, paired t test.

**Figure 3.** CD39-expressing, IL-10-secreting CD8<sup>+</sup> Treg cells suppress proliferation of naïve T cells in a CD39-dependent fashion. (A&B) OT-I CD8<sup>+</sup> cells were differentiated as in Figure 1 into Tc1 cells (peptide alone, expressing no CD39 or IL-10) or Treg cells (peptide + IL-4 + IL-12, expressing CD39 and IL-10). (A) These were added to freshly isolated OT-I cells labelled with CFSE fluorescent marker, with or without addition of ARL67156 CD39 inhibitor. After 3 days cellular division of target cells was assessed by dilution of CFSE: (Left) Dose:response curve comparing suppressive activity of Tc1 cells to Treg cells at different ratios. (Right) % divided cells in the CFSE<sup>+</sup> CD8<sup>+</sup> gate is shown. Data are shown as mean+SEM (n=4) and are pooled from 4 independent experiments using a 1:4 effector:target ratio. \*:p<0.05, \*\*\*: p<0.0005, paired t test. (B) ATP/ADP breakdown (first 2 graphs), AMP release (third graph) from day 5 Tc1 and Treg cells pulsed with ATP, and inosine release from adenosine-pulsed Tc1 and Treg cells (fourth graph). Data are shown as mean+SEM (n=4 for left 3 graphs, n=3 for right graph) and are pooled from 3 or 4 independent experiments. \*: p<0.05; \*\*: p<0.005; 2-way ANOVA.

**Figure 4.** Induction of CD39 in vivo during development of T-cell tolerance. (A-C) OT-I mice were injected i.p. with OVA<sup>257</sup> peptide or PBS control on days 0,1,2,3,4, & 7. OT-I mice were bled and CD39 expression on circulating CD8 cells measured by flow cytometry. (A) Circulating CD8 T cells were stained for CD39 and CD44 at

different time points of tolerization. Cells were gated on CD8 $\beta$ <sup>+</sup> events; representative staining from 3 independent experiments. (B) Pooled data showing % CD39<sup>+</sup>, CD44<sup>hi</sup>, CD45RB<sup>lo</sup> and CD62L<sup>+</sup> phenotypes as measured in CD8 $\beta$ <sup>+</sup> cells (day 10) by flow cytometry. Data from n=8-12 mice pooled from 3 independent experiments are shown. (C) Proliferative responses of splenocytes from naïve (control) or peptide-primed mice (day 10, “tolerized”), in response to OVA<sup>257</sup> peptide in vitro, expressed as % divided within CFSE<sup>+</sup> gate. Data from 4 mice pooled from 2 independent experiments (mean+SEM) shown. \*\*: p<0.005; \*\*\*: p<0.0005, unpaired t test; \*\*\*\*: p<0.00005, paired t test; NS: not significant.

**Figure 5.** Induction of CD39 in CD8<sup>+</sup> T-cell tolerance involves IL-12. OT-I mice were tolerized to OVA<sup>257</sup> peptide as in Figure 4, with injection of anti-IL-12 antibody or rat IgG control on days 0, 2 and 4. (A) CD39 expression on circulating CD8<sup>+</sup> T cells; n=6 mice per group, pooled from 2 independent experiments, mean+SEM shown. (B) Intracellular staining for T-bet and Foxp3 in blood CD8<sup>+</sup> T cells from mice as in A (day 10). (C) IL-12 p70 and IL-10 levels in serum from control or tolerized OT-I mice. Data are from 8-12 mice pooled from 3 independent experiments, mean+SEM. \*: statistical comparisons between OVA<sup>257</sup>+ IgG and PBS group; †: comparison of IgG vs anti-IL-12 groups. \*: p<0.05; \*\*\*: p<0.0005, †: p<0.05, unpaired t test.

**Figure 6.** An A2A agonist enhances CD39 expression on CD8 T cells during tolerization. OT-I mice were tolerized as in Figure 4 with or without co-injection of CGS21680 (A2A adenosine receptor agonist) or diluent control. CD39 expression on blood CD8<sup>+</sup> T cells was tracked. Data are shown as mean+SEM (n=8) and were pooled from 2 independent experiments. Statistical comparisons between CGS21680 and diluent control groups are indicated. \*: p<0.05; \*\*\*: p<0.0005, unpaired t test.

**Figure 7.** (A&B) Tolerance to inhaled allergen involves recruitment of CD39-expressing CD8<sup>+</sup> T cells to the lungs and airways. For tolerance induction C57BL/6 mice were given PBS alone or OVA+PBS intranasally. (A) Total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in bronchoalveolar lavage (BAL) and their expression of CD39 measured by flow cytometry on day 6. Data are mean+SEM (n=8) and are pooled from 2 independent experiments. (B) Expression of CD39 on CD8<sup>+</sup> T cells extracted from lung tissue or draining lymph nodes (LN), data are mean+SEM data (n=8 mice) pooled from 2 independent experiments. (C) Tumor-infiltrating lymphocytes (TIL) include CD8<sup>+</sup> CD39<sup>+</sup> cells: B16.OVA melanoma cells were injected s.c. into C57BL/6 mice and one month later tumors were excised and TIL extracted. CD39 expression on TIL and control inguinal lymph node cells is shown as mean+SEM (n=6 mice), data are pooled from 2 independent experiments. \*\*:p<0.005; \*\*\*: p<0.0005; \*\*\*\*: p<0.00005, Mann Whitney tests.

Figure 1

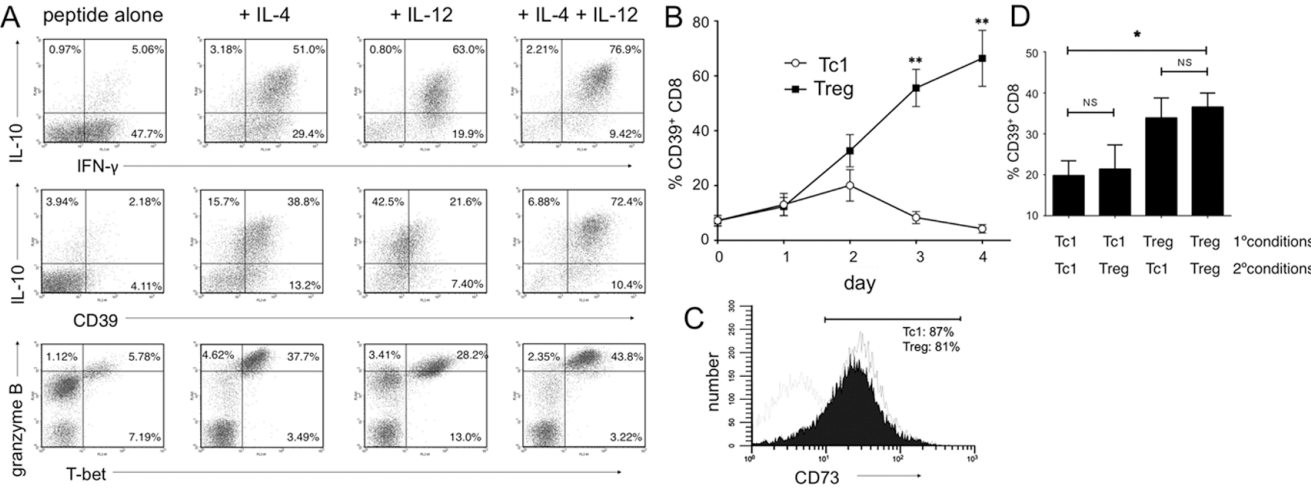


Figure 2

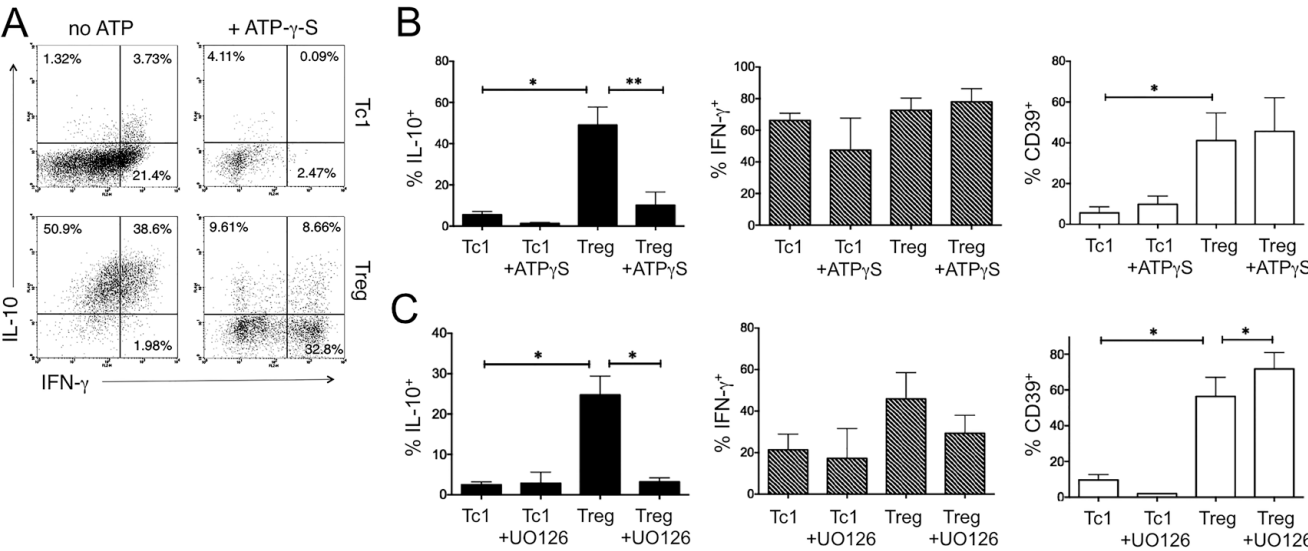




Figure 3

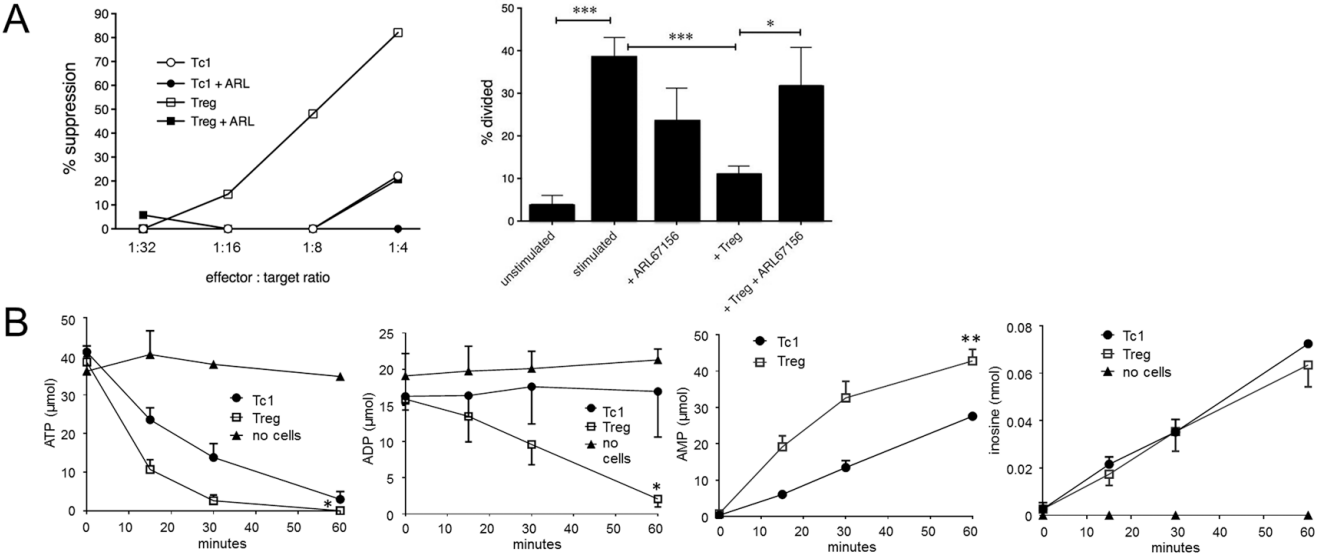


Figure 4

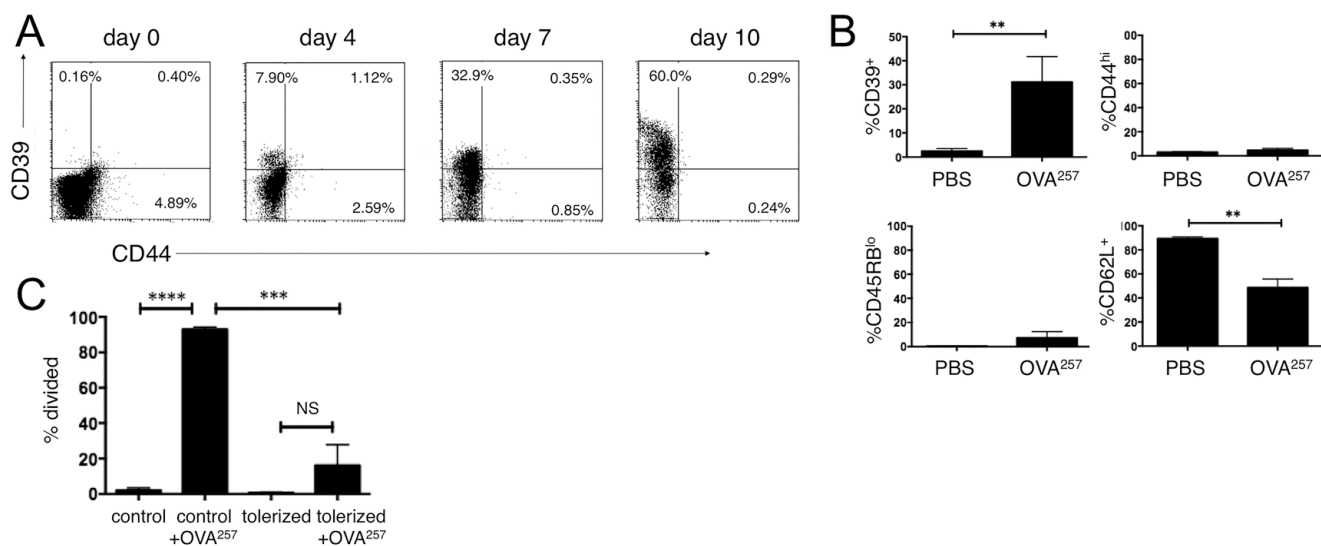


Figure 5

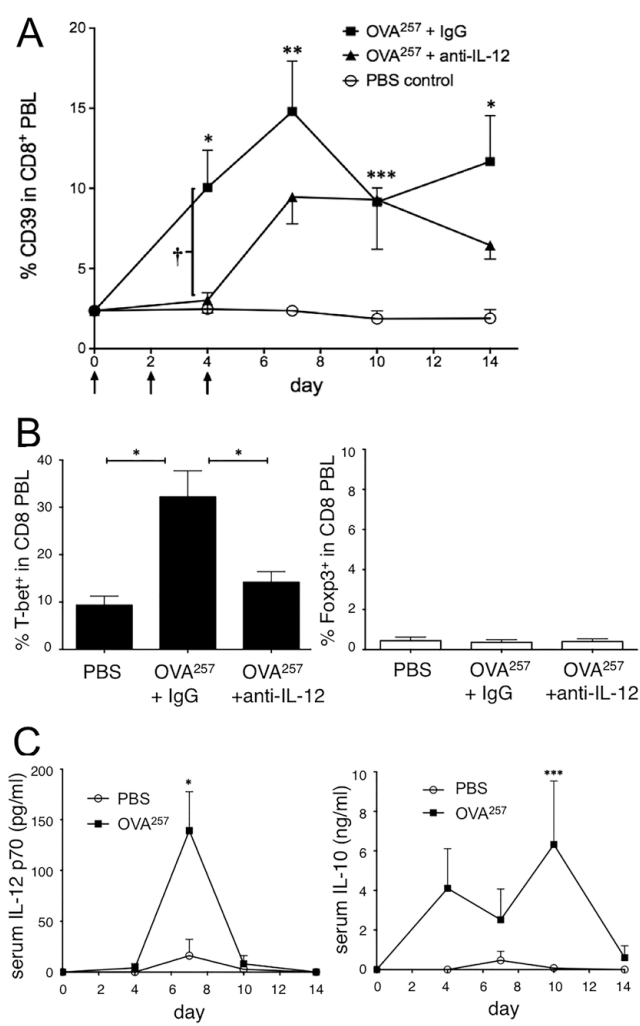


Figure 6

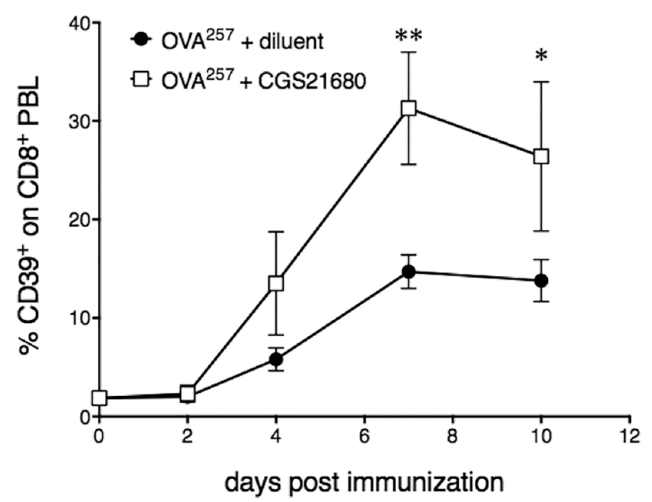
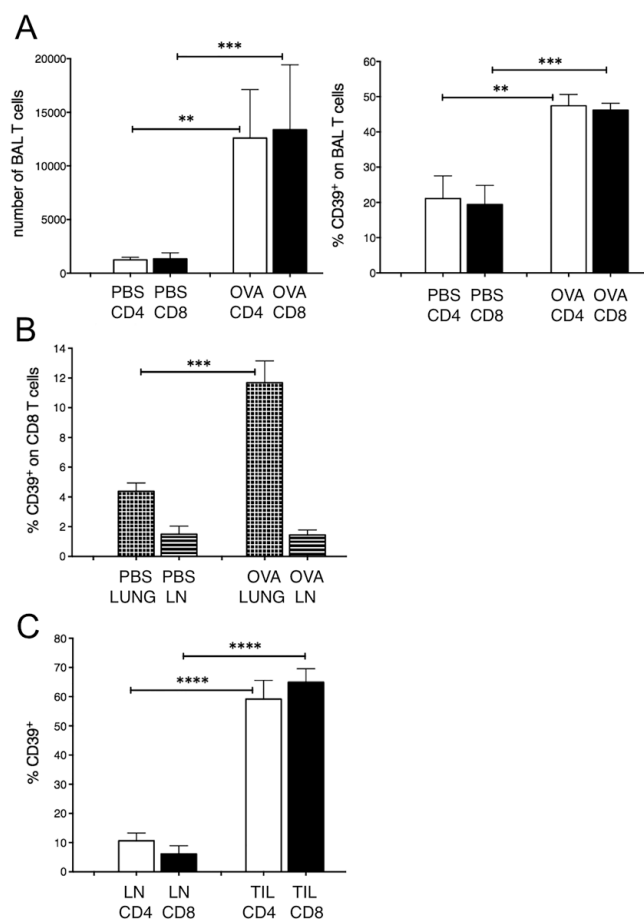


Figure 7



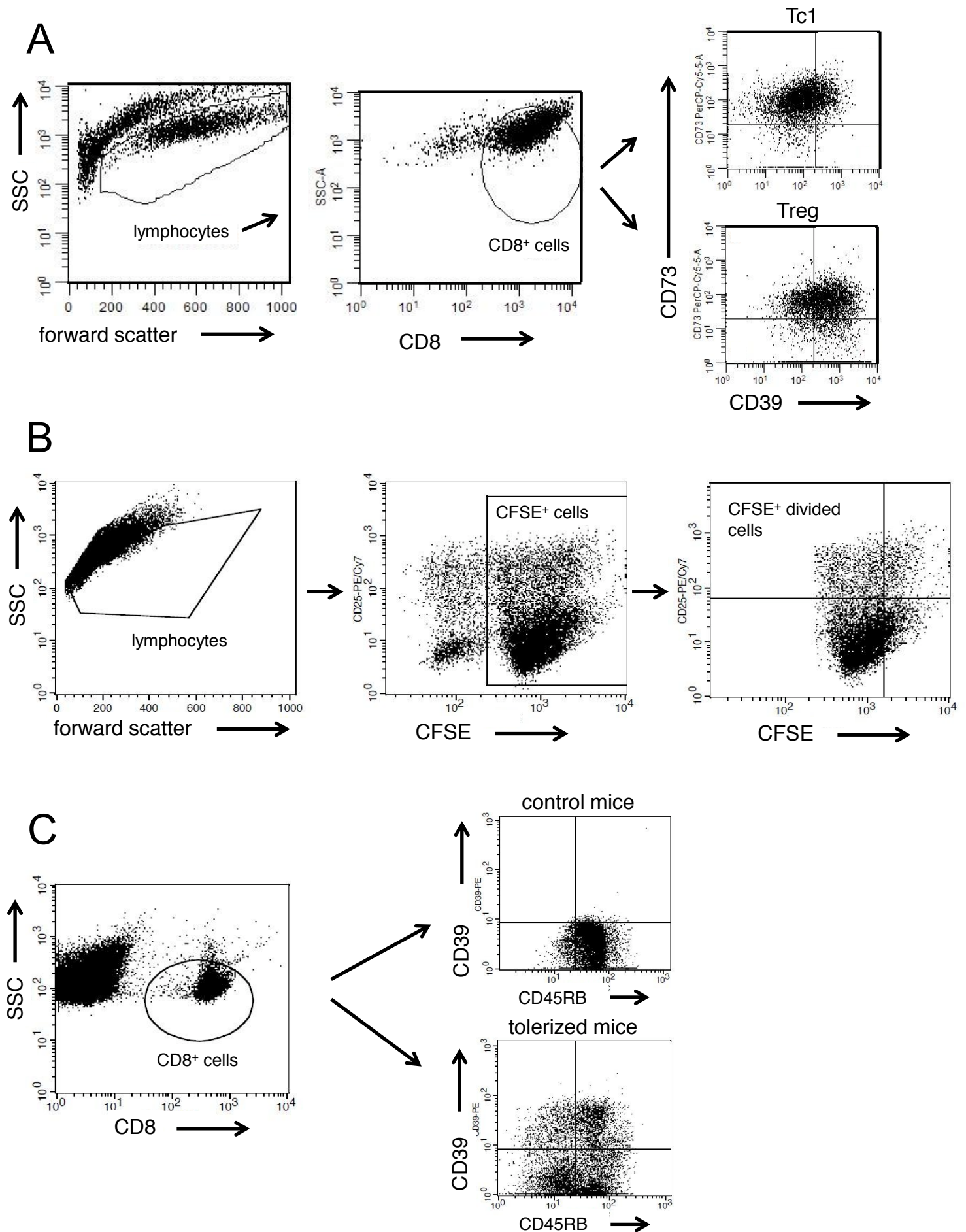


Figure S1, online supplement. A: Gating strategy used in flow cytometric analysis of in vitro-generated Tc1 and Treg CD8 cells (Figures 1&2). B: Gating used in suppression assays (Figure 3A) to distinguish target (CFSE<sup>+</sup>) from effector (CFSE<sup>-</sup>) cells. C: Gating for CD39 & CD45RB expression on peripheral blood CD8 T cells used in Figures 4, 5&6.

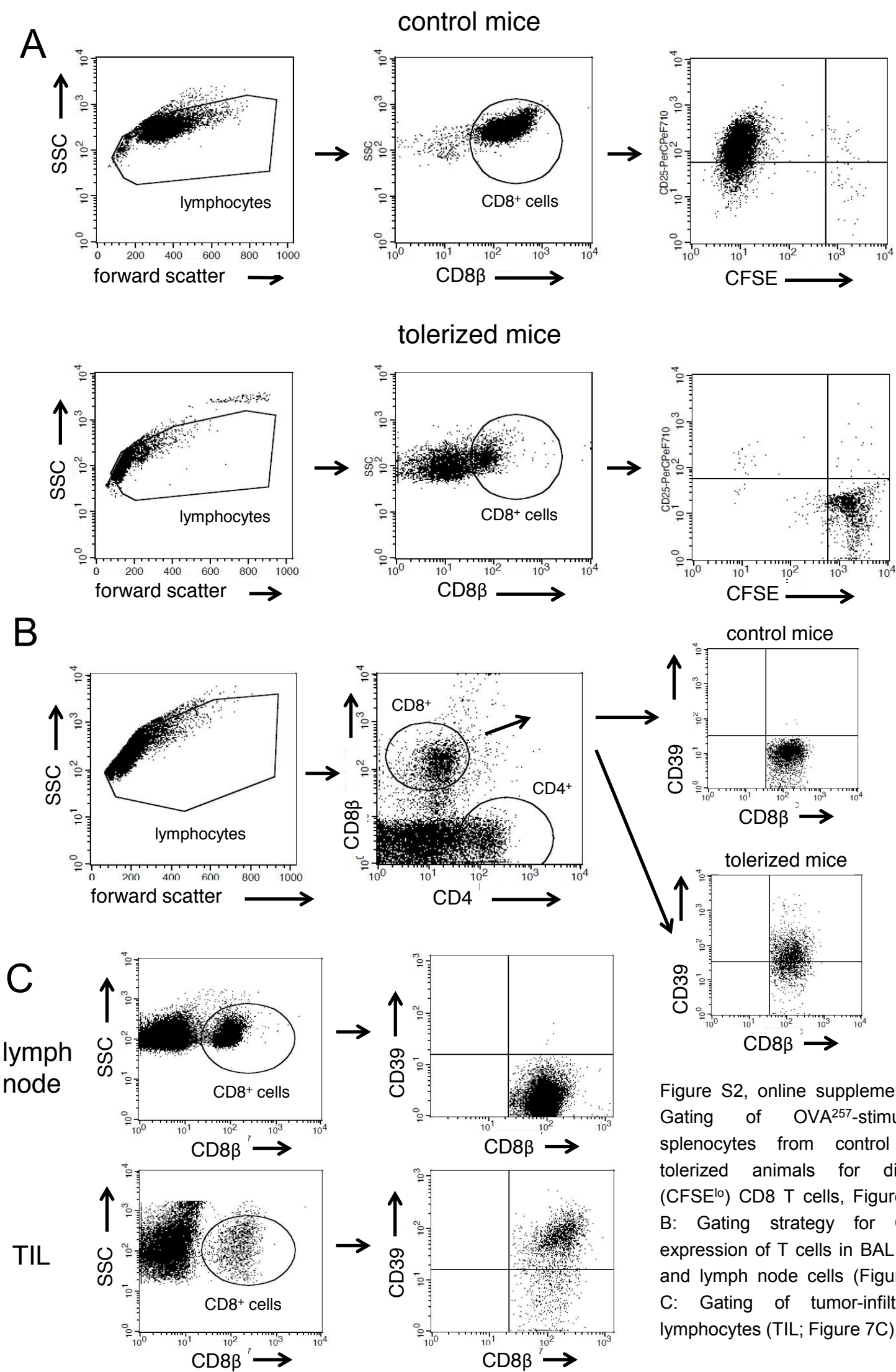


Figure S2, online supplement. A: Gating of OVA<sup>257</sup>-stimulated splenocytes from control and tolerized animals for divided (CFSE<sup>lo</sup>) CD8 T cells, Figure 4C. B: Gating strategy for CD39 expression of T cells in BAL, lung and lymph node cells (Figure 7). C: Gating of tumor-infiltrating lymphocytes (TIL; Figure 7C).